Life Sciences, Vol. 66, No. 23, pp. 2255-2260, 2000

0024-3205/00/$-see front matter

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PII S0024-3205(00)00554-3

INDUCTION OF APOPTOSIS IN MOUSE BRAIN CAPILLARY ENDOTHELIAL CELLS BY CYCLOSPORIN A AND TACROLIMUS

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(Received in final form January 5, 2000)

Summary

Although cyclosporin A and tacrolimus are used clinically as potent immunosuppressants, there have been reports of neurotoxicity and encephalopathy. A possible mechanism is that these drugs damage the blood-brain barrier (BBB), inducing dysfunction and increased permeability, and are then able to enter the brain. We studied the cytotoxicity of cyclosporin A and tacrolimus, focused on apoptosis induction, using an immortalized cell line established from BALB/c mouse cerebral microvessel endothelial cells (MBEC4). We found that these two drugs induced cell shrinkage, chromatin condensation and DNA fragmentation, which are characteristics of apoptosis. Our data suggest that the induction of apoptosis on the brain capillary endothelial cells may be at least partly involved in the occurrence of immunosuppressant-induced encephalopathy.

Key Words: encephalopathy, cyclosporin A, tacrolimus, blood-brain barrier, apoptosis

The cyclic polypeptide cyclosporin A and the cyclic macrolide tacrolimus are two potent immunosuppressants used in organ transplantation to prevent the fetal graft-versus-host disease or to suppress the rejection symptoms (1, 2). They act by binding to the immunophilins and inhibiting transcription of Il-2, the major T-cell growth factor (3). These two drugs induce the apoptotic cell death of T-cells, B-cells and thymocytes and this may contribute to their immunosuppressive effects (4-6).

Cyclosporin A and tacrolimus are evaluated highly in clinical field as potent immunosuppressants, however they induce acute and chronic nephrotoxicity (7, 8). Moreover, encephalopathies such as seizure, confusion, motor paralysis and cortical blindness have been reported in transplant patients using these drugs (9, 10). The mechanism of the immunosuppressant-induced encephalopathy is still unclear, though patients who developed encephalopathy had edema of the brain usually in the cerebral white matter. It is considered that the toxic effects of the drugs to the nerve cells are the terminal factor of immunosuppressant-induced encephalopathy. However, in order to act on the nerve cells, the drugs must permeate blood-brain barrier (BBB) and enter into the brain. The

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transport of these two drugs across the BBB is restricted by P-glycoprotein (P-gp) and consequently their accumulation in the brain is low (11, 12). It is speculated that the encephalopathy is induced as a result of enhanced brain distribution of the drugs due to increased permeation across the BBB arising from injury to brain capillary endothelial cells that form the BBB and/or depressed function of P-gp at the BBB. Recently, we have reported that the effects of cyclosporin A and tacrolimus on the cellular viability and P-gp function in an immortalized cell line established from BALB/c mouse cerebral microvessel endothelial cells (MBEC4): 1) the drugs induced increased permeability to [^{3}H]sucrose and the release of LDH, 2) they decreased cellular viability, 3) the uptake of [^{3}H] vincristine, a substrate of P-gp, was increased, 4) P-gp expression was reduced. Our findings suggested that these two drugs damaged the barrier function of brain capillary endothelial cells by direct cytotoxic action and inhibited the function of P-gp. It has also been reported that cyclosporin A inhibited cellular growth and induced apoptosis characterized by chromatin condensation, DNA fragmentation and shrinkage of the cell body in rat C6 glioma cells in a dose-dependent manner. It has been suggested that the induction of apoptosis in nerve cells might contribute to the neurotoxicity of cyclosporin A (13). Therefore, we thought that there was a possibility that cyclosporin A and tacrolimus also induce apoptosis in brain capillary endothelial cells and interference of the BBB function.

To investigate the possible mechanism of immunosuppressant-induced encephalopathy, we studied the toxic effects of cyclosporin A and tacrolimus on brain capillary endothelial cells using MBEC4 (14, 15) and examined the contribution of apoptosis induction to their cytotoxicity as one of the underlying mechanisms.

Material and Methods

Reagents Cyclosporin A and tacrolimus were kindly supplied by Novartis Pharma (Basel, Switzerland) and Fujisawa Pharmaceutical Inc. (Osaka, Japan), respectively. Hoechst 33342 was purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). ϕX174/Hinc II digest and λ/Hind III digest were purchased from Toyobo Co. Ltd. (Osaka, Japan). All other chemicals were commercial products of reagent grade. Cyclosporin A and tacrolimus were dissolved in ethanol and diluted in serum-free culture medium (0.1% final ethanol concentration).

Cell cultures Mouse brain capillary endothelial cell line (MBEC4), derived isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation (14), was cultured in Dulbecco's modified Eagle's medium (Nikken Bio Medical Lab, Japan) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells (4 x 10^4 cells/ml) were cultured in 24-well Collagen type I plates (Iwaki Glass, Chiba, Japan) in a humidified atmosphere of CO_2/air (5% / 95%). After 3 days of culture, the cells were washed 3 times with serum-free medium and treated with various concentrations of cyclosporin A or tacrolimus for 12 or 24 h. In parallel, cells were treated with serum-free medium containing the corresponding amount of ethanol as the control. MBEC4 cells retain the morphological and biochemical characteristics of brain capillary endothelial cells and express P-gp mainly encoded by the *mdr1b* gene (14, 15), so they are considered a useful and convenient model to evaluate the specific effect of drugs at the BBB.

Assessment of morphological changes MBEC4 were treated with 1 and 10 μM cyclosporin A or tacrolimus for 12 h, and the morphological changes were observed under a phase-contrast microscope (Olympus, IX70).

Nuclear staining of DNA Nuclear staining was performed as described previously (16).

MBEC4 cells grown on cover glass coverslip were treated with the vehicle alone or 1 or 10 μM cyclosporin A or tacrolimus f r 12 h. They were fixed for 30 min in a solution containing 4% paraformaldehyde in PBS and then exposed to 0.5 mM Hoechst 33342 dye for 30 min. Cultures were washed with PBS three times. Cells were observed under a fluorescence microscope (LEICA DMRB) with excitation at 340 nm.

DNA fragment assay MBEC4 were treated with various concentrations of cyclosporin A or tacrolimus for 24 h and lysed in lysate buffer (10 mM Tris-HCl, 10 mM EDTA; pH 8.0, 10% Triton X-100). After centrifugation at 16000 rpm for 20 min, 2 μl of RNase A (20 mg/ml) was added to the supernatant and the mixture was incubated at 37℃ for 1 h. Then, 2 μl of Proteinase K (20 mg/ml) was added to the mixture and incubation was continued at 37 ℃ for 1 h. NaCl and isopropanol were added to concentrations of 0.5 M and 50%, respectively and the mixture was left at -20℃ overnight. After centrifugation at 16000 rpm for 15 min, the pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA). The samples were subjected to electrophoresis on 2% agarose gel and the gel was stained with ethidium bromide.

Results

Morphological effects of cyclosporin A or tacrolimus on MBEC4 The control cells treated with serum-free medium for 12 h, showed the characteristic features of brain capillary endothelial cells, such as spindle-shape and tight junctions with neighboring cells (Fig. 1A). On the other hand, 1 and 10 μM cyclosporin A- or tacrolimus-treated cells became rounded-up and detached from the substratum. The alterations seemed to be a little more marked in tacrolimus-treated cells compared with the cyclosporin A-treated cells, but there were no large differences between the results of cyclosporin A treatment and tacrolimus treatment at the concentrations used in this experiment.

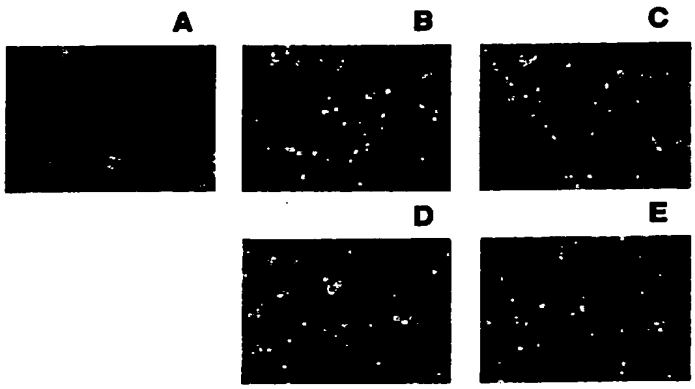

Fig. 1
Morphological changes in MBEC4 cells induced by treatment with cyclosporin A or tacrolimus for 12 h
A: control cells, B: 1 μM cyclosporin A-treated cells, C: 10 μM cyclosporin A-treated cells, D: 1 μM tacrolimus-treated cells, E: 10 μM tacrolimus-treated cells

Effects of cyclosporin A and tacrolimus on nuclear DNA The specific DNA fluorescence stain Hoechst 33342 was used to assess the alterations in DNA and nuclear structure following treatment with 1 and 10 μM cyclosporin A or tacrolimus for 12 h. As shown in Fig. 2, the nuclei of cells treated with cyclosporin A or tacrolimus underwent condensation and fragmentation of chromatin. Such changes were not seen in control cells.

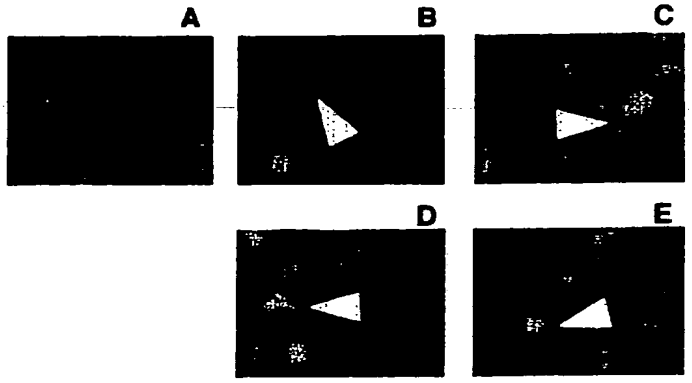

Fig. 2

Alterations in nuclear morphology induced by cyclosporin A or tacrolimus in MBEC4 cells. Cultures were treated with serum free-medium alone (A), 1 μM (B) or 10 μM (C) cyclosporin A or 1 μM (D) or 10 μM (E) tacrolimus for 12 h and stained with Hoechst 33342. Arrows show chromatin condensation in nuclei of MBEC4 cells.

Cyclosporin A and tacrolimus-induced DNA fragmentation Fig. 3 shows the electrophoretogram of DNA extracted from control cells and cells treated with cyclosporin A or tacrolimus for 24 h. DNA isolated from cells treated with 0.1, 1 and 10 μM cyclosporin A or tacrolimus exhibited a ladder-like pattern of DNA fragmentation into oligonucleosome-length fragments.

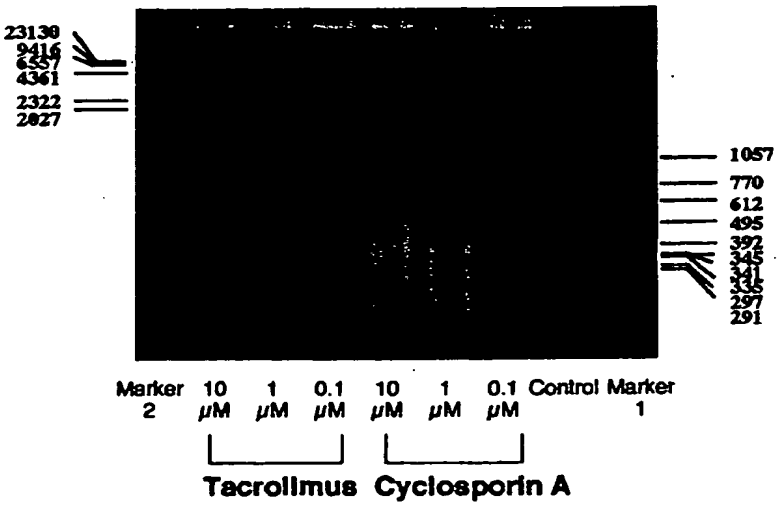

Fig. 3

Ladder-like DNA fragmentation in MBEC4 cells induced by treatment with cyclosporin A or tacrolimus for 24 h. Marker 1; ϕX174/Hinc II and Marker 2; λ/Hind III (DNA molecular weight standards)

Discussion

Apoptosis is a highly conserved mode of cell death that is essential for normal development and host defense. It has also been implicated in the occurrence of diseases such as cancer, Alzheimer's disease and AIDS, and in the induction of adverse drug effects (13, 16-18).

Recently, it was reported that cyclosporin A and tacrolimus, potent immunosuppressants, can induce encephalopathy (9, 10). Because these drugs are actively transported out of the brain by P-gp (11, 12), neurotoxicity is unexpected. A possible mechanism is that these drugs induce increased permeability of the BBB. Indeed, our previous study indicated that they inhibit P-gp function and reduce P-gp expression through direct cytotoxicity to brain capillary endothelial cells. In the present study, we investigated whether these two drugs induce apoptosis in brain capillary endothelial cells MBEC4 *in vitro*, in order to elucidate the underlying mechanism of their cytotoxic effects.

We found that these two drugs induced the following alterations in MBEC4; 1) cells growing in monolayer became rounded-up with loss of junctions with neighboring cells, and detached from the substratum; 2) cell nuclei underwent condensation and fragmentation of chromatin; 3) DNA isolated from treated cells was fragmented into oligonucleosome-length fragments. These alterations are characteristic features of apoptosis. It appears that these two drugs do induce apoptosis in MBEC4. However, there was no marked difference between the effects of cyclosporin A and tacrolimus. Moreover, dose-dependency was not observed in the range of concentrations used in this study. Therefore, it is possible that much lower drug concentrations might be possible to induce apoptosis. In MBEC4 cells, it is conceivable that p53 protein-related apoptosis may be suppressed by SV40 large T antigen, whose gene was transfected to confer the immortality on the mouse brain capillary endothelial cells. Even under overexpression of SV40, apoptosis was observed in MBEC4 cells. Therefore, apoptosis may be induced in normal brain capillary endothelial cells with higher possibility. However, it also should be taken into consideration, and will be discussed later, that the drug concentrations used in this study were quite high in comparison with their clinical range.

We have also reported previously that cyclosporin A and tacrolimus increase the paracellular permeability and the release of LDH in MBEC4. Therefore, we speculated that these two drugs induce increased permeability of MBEC4 monolayer and injury of cellular membranes and thereby enhance the permeability of the BBB. In addition, we showed here that cyclosporin A and tacrolimus induce apoptosis in MBEC4. These findings suggest that a direct cytotoxic effect, an inhibitory effect on P-gp function and the induction of apoptosis may all involved in the enhancement of permeability of the BBB. Further studies in a lower drug concentration range and with a longer duration of exposure are needed, because the drug concentrations used in this study were extremely high compared with these used in the clinical field (cyclosporin A; 100-300 nM, tacrolimus; 6-24 nM). When the plasma protein binding (cyclosporin A; 98.4% (19), tacrolimus; 78% (20)) and the plasma/blood ratio (cyclosporin A; 0.4 (21), tacrolimus; 0.1 (22)) are taken into consideration, the clinical unbound concentrations of cyclosporin A and tacrolimus in plasma are estimated to be 0.6-1.9 and 0.5-2.0 nM, respectively. In this study, we used serum-free medium as the vehicle to exactly regulate the drug concentrations. However, culture under serum-free conditions for over 24 h damaged MBEC4, so we performed the experiments within 24 h and were obliged to observe toxic effects within this short period instead of chronic exposure. Moreover, MBEC4 may be conceivably insusceptible to drug-induced apoptosis because of the overexpression of SV40 antigen. These facts obliged us to use quite high concentration of drugs.

The summary, our results indicate that cyclosporin A and tacrolimus induce apoptosis, characterized by cell shrinkage, chromatin condensation and DNA fragmentation, in MBEC4 cells. Thus, the damage to brain capillary endothelial cells, leading to increased permeability of the BBB may be involved,at least in part, in the mechanism of immunosuppressant-induced encephalopathy.

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